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TECHNICAL MANUSCRIPT 435

A PLAQUE PROCEDURE  
FOR THE ASSAY OF RICKETTSIA RICKETTSII

Edmund H. Weinberg  
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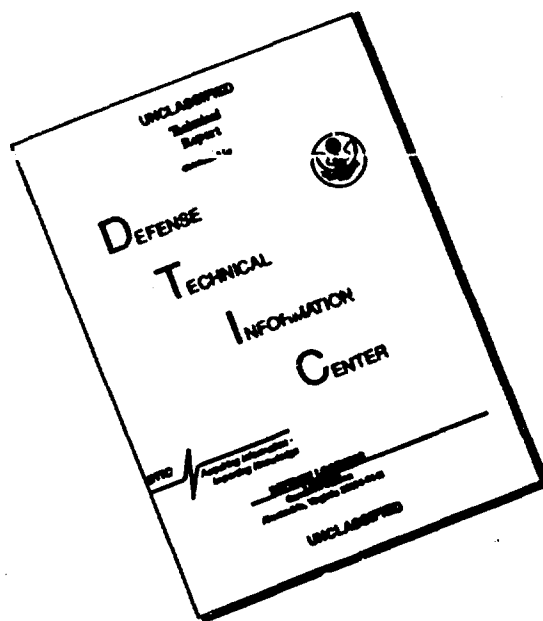
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TECHNICAL MANUSCRIPT 435

A PLAQUE PROCEDURE FOR THE ASSAY OF RICKETTSIA RICKETTSII

Edmund H. Weinberg

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BIOLOGICAL SCIENCES LABORATORY

Project 1C522301A059

April 1968

### ABSTRACT

A plaque technique for the assay of Rickettsia rickettsii is described. The method employs primary chick fibroblast cell cultures with a semisolid agar overlay. Plaques were observed after 6 days' incubation, and the titers correlated well with those obtained in embryonated eggs infected by the yolk-sac route. Identification of the rickettsia as the plaque-forming organism was accomplished by direct observation of rickettsial-like bodies in the monolayer lesions, specific fluorescent antibody staining, inhibition of plaques by antibiotics, sensitivity of the plaque to specific immune serum, and failure to cultivate, by a variety of methods, other microorganisms from the infected cells.

## I. INTRODUCTION

It is well recognized that laboratory research with rickettsiae has been impeded by the lack of a suitable assay system to substitute for the usual method of titration in embryonated eggs. In 1966, Kordova\* described a plaque technique used with several species of rickettsiae. The sensitivity of her plaque test was 100-fold lower than the yolk-sac method, a period of 14 to 17 days was required for completion of the test, and a high percentage of experiments resulted in unexplained failures. The major objective of our research was to develop a plaque assay procedure for Rickettsia rickettsii that would be as sensitive as and require less time than the titration in embryonated eggs. Preliminary observations showed that chick embryo primary cells were infected and a cytopathic effect (cpe) was produced by R. rickettsii when the cell monolayers were under a liquid overlay. In contrast, no appreciable cpe was noted when a 1% agar overlay was used. The purpose of this paper is to describe the observations that led to the development of a plaque assay employing a semisolid agar overlay.

## II. MATERIALS AND METHODS

The organism studied was the Bitterroot strain of R. rickettsii, eighth passage in chick embryo yolk sacs. The working seed had a titer of  $1 \times 10^{7.4}$  YSLD<sub>50</sub>/ml.

Ten-day-old chick embryo cells, dispersed by mincing and trypsinizing, were used to produce the primary monolayer cell sheets used for the plaque formation experiments. The nutritive medium was Nagle's chemically defined medium with 5% inactivated calf serum, and the cells were grown in 30-ml plastic bottles with the caps tightly closed. All cultures were incubated at 37 C until confluent monolayers were available (24 hours), the medium was decanted, and the cells were infected with R. rickettsii. The inoculum, consisting of freshly thawed yolk-sac suspensions of rickettsiae diluted in cold Nagle's medium, was placed on the cell sheets in a volume of 0.1 ml. After adsorption at 25 C for 90 minutes, a 5-ml volume of Nagle's medium, containing 5% inactivated calf serum and 0.15% Noble special agar, was applied as a semisolid overlay, and the cultures were incubated at 32 C for 6 days. The agar overlay was removed by inclining the bottle and gently tapping the sides. Staining was accomplished by placing 5 ml of an isotonic neutral red solution on the cells for 15 minutes at 37 C. Thirty minutes after removal of the staining solution, plaques were observed. A standard radiograph viewer provided the illumination for such observations.

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\* Kordova, N. 1966. Plaque assay of rickettsiae. *Acta Virol.* 10:278.

### III. RESULTS

Figure 1 shows three bottles with plaques resulting from three 10-fold dilutions ( $1 \times 10^{-4}$ ,  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$ ) of the original suspension that contained  $1 \times 10^{7.4}$  YSLD<sub>50</sub>/ml. The plaques were quite uniform in size, averaging 1.5 mm in diameter. A 10-fold dilution of the inoculum resulted in the expected 10-fold decrease in plaque numbers (Table 1). Microscopically, the cells surrounding the plaque appeared normal, but the cells in each plaque were rounded, granular, and easily detached from the plastic surface. When stained with carbolfuchsin followed by malachite green, numerous intracytoplasmic rods consistent in morphology with R. rickettsii were seen. No rods were seen within the cytoplasm of cells outside the plaque, although an occasional extracellular organism was seen in the supernatant fluid. Very few cells were observed to be actually lysed when the overlay medium was centrifuged and examined. When a mixture of antibiotics including streptomycin, chlortetracycline, penicillin, and Mycostatin was incorporated into the overlay, no plaques were obtained at any dilution.

TABLE 1. COUNTS OF PLAQUES RESULTING FROM INFECTION OF CHICK PRIMARY MONOLAYER CELLS WITH RICKETTSIA RICKETTSII

Dilution of Inoculum <sup>a</sup>	Plaque Count	Mean $\times 10^7$
$1 \times 10^{-7}$	2; 4; 1	2.3
$1 \times 10^{-6}$	21; 20; 24	2.17
$1 \times 10^{-5}$	164	1.64

a. Tissue culture inoculum 1 ml of suspension  $1 \times 10^{7.4}$  YSLD<sub>50</sub>/ml ( $\log_{10}$ ).

Figure 2 shows the cell sheets obtained when antibiotics were present in the overlay during incubation. These bottles were part of the same experiment shown in Figure 1. When 0.5 ml of specific immune serum of human origin having a complement fixation titer of 1:512 was placed in the overlay medium of each bottle, the plaque size was reduced to an average of 0.5 to 0.75 mm, and the plaque borders were no longer uniform and sharply demarcated. The plaque numbers, however, were not reduced.

Additional sources of inoculum also succeeded in producing plaques about equal to their YSLD<sub>50</sub> titers. Further evidence that the plaques resulted from R. rickettsii was obtained when aerobic and microaerophilic (10% CO<sub>2</sub>) cultures using brain heart infusion broth, thioglycollate broth, Nagle's medium, PFLO broth, and agar failed to demonstrate bacterial, fungal, and PFLO growth when cells obtained from a plaque were used as the inoculum. The intracellular rickettsiae were also stained by a direct fluorescent antibody technique.

#### IV. CONCLUSION

We believe we have developed a useful plaque assay technique for R. rickettsii. It is important to stress that the agar concentration appears to be critical in permitting good quality plaques that can be produced in as little as 6 days. As an assay system, the procedure seems to be at least as sensitive as the yolk-sac titration method and of course offers numerous advantages over it as a means to study the organism on a more critical level. We expect to determine the applicability of this test to other rickettsiae.





Figure 1. Plaques Resulting from 10-fold Dilutions of a Suspension of Rickettsia rickettsii Strain R1 on Chick Primary Monolayer Cells. An uninoculated control appears on the far left.

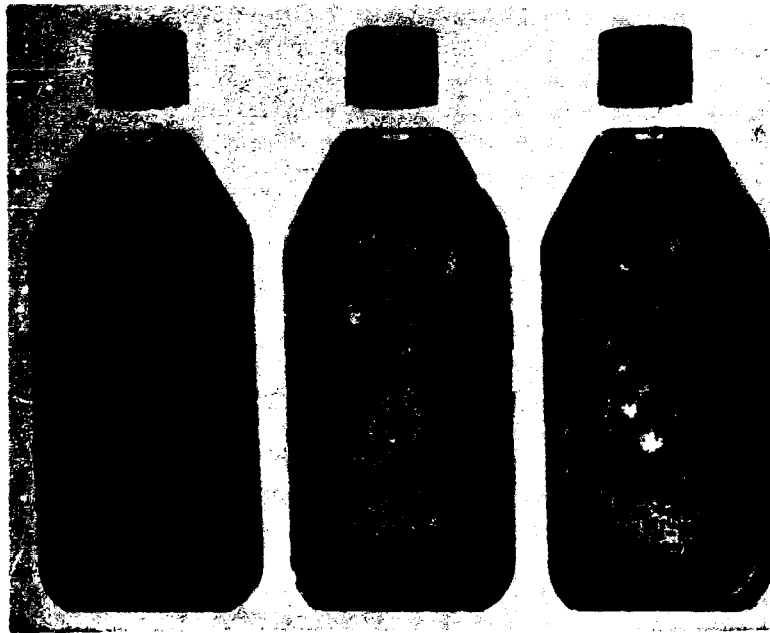


Figure 2. Suppression of Plaques in Bottles Containing Three 10-fold Dilutions of Rickettsia rickettsii when Penicillin, Streptomycin, Chlorotetracycline, and Mycostatin are Incorporated in the Agar Overlay.

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